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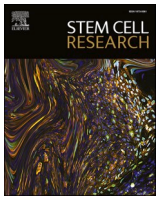

Generation of a human embryonic stem cell reporter line, *TMEM119*-EGFP, for the visualisation of *in vitro* differentiated human microglia

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A B S T R A C T

Transmembrane protein 119 (TMEM119) is a recently identified microglia marker that is not expressed by other immune cells. Using CRISPR/Cas9 technology, we introduced enhanced green fluorescence protein (EGFP), into the H9 WA-09 human embryonic stem cell line, directly before the *TMEM119* stop codon. Sanger sequencing confirmed successful insertion of the EGFP sequence. The newly created cell line expressed a normal morphology and karyotype, several pluripotency markers, and the ability to differentiate into all three germ layers. H9-*TMEM119*-EGFP can be used to provide a deeper understanding of the role of *TMEM119* in microglia by monitoring its expression under different experimental conditions.

Resource Table:

Unique stem cell line identifier	WAe009-A-D
Alternative name(s) of stem cell line	H9-*TMEM119*-EGFP
Institution	Ajou University School of Medicine
Contact information of the reported cell line distributor	Junghyun Jo, junghyunjo@ajou.ac.kr
Type of cell line	Embryonic stem cells (ESCs)
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	WA09 (H9 ESC)
Cell Source	N/A
Method of reprogramming	N/A
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
Cell culture system used	mTeSR, Matrigel coated plates (1:30 Matrigel:DMEM/F12 dilution), 37 °C, 5 % CO_2 Feeder-free culture system
Type of Genetic Modification	CRISPR/Cas9 excision and insertion of fluorescent reporter
Associated disease	N/A
Gene/locus	*TMEM119*/12q23.3
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Plasmid transfection/electroporation
All genetic material introduced into the cells	Cas9 plasmid, donor plasmid, EGFP plasmid
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele
Method of the off-target nuclease activity surveillance	Targeted PCR and sequencing
Name of transgene	Enhanced green fluorescence protein (EGFP)

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	A visual record of the line's cellular morphology: normal	Fig. 1 panel G
Pluripotency status evidence for the described cell line	Qualitative analysis (immunocytochemistry)	Positive for pluripotency markers Oct4, NANOG	Fig. 1 panel E
	Quantitative analysis (i.e. Flow cytometry, RT-qPCR)	Not performed	N/A
Karyotype	Karyotype (G-banding)	46XX, >500 bhps	Fig. 1 panel D
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	PCR specific to desired KI (junction sequencing)	Fig. 1 panel B/C
	Transgene-specific PCR	Not performed	N/A
Verification of the absence of random plasmid integration events	PCR/Southern	PCR detection specific for plasmid backbones	Supplementary Fig. 2
Parental and modified cell line genetic identity evidence	STR analysis	10 sites tested: amelogenin + 9 loci. 100 % match.	Submitted in the archive with the journal
Mutagenesis / genetic modification outcome analysis	Sequencing	Homozygous	Fig. 1 panel C
	PCR-based analyses	Homozygous target integration	Fig. 1 panel B
	Southern Blot or WGS	Not performed	N/A
Off-target nuclease analysis-	PCR across top 8 predicted top likely off-target sites	Undetected	Supplementary Fig. 1
Specific pathogen-free status	Mycoplasma	Negative	Supplementary Fig. 3
Multilineage differentiation potential	2D spontaneous differentiation	Immunocytochemistry	Fig. 1 panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	N/A
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

(*continued*)

Unique stem cell line identifier	WAe009-A-D
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	Puromycin
Inducible/constitutive system details	N/A
Date archived/stock date	April 2021
Cell line repository/bank	https://hpscreg.eu/cell-line/WAe009-A-D
Ethical/GMO work approvals	The cell line was generated according to institutional guidelines.
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52,961; https://n2t.net/addgene:52961; RRID: Addgene_52961) pUC19 was a gift from Joachim Messing (Addgene plasmid # 50,005; https://n2t.net/addgene:50005; RRID: Addgene_50005)

1. Resource Utility

TMEM119 has been identified as a transmembrane protein expressed by microglia but not other macrogphages. The H9-*TMEM119*-EGFP reporter cell line can be used to differentiate human embryonic stem cells (hESCs) into microglia. This EGFP-reporter cell line provides an invaluable genetic tool for studying microglia in human physiology and disease (Table 1).

2. Resource details

Studies have revealed that several microglial markers were also expressed by peripheral macrophages and other immune cells owing to their shared lineage derivation. However, single-cell RNA sequencing revealed that *TMEM119* is a microglia-specific marker in both humans and mice and is not expressed by any other immune cell (Bennett et al., 2016). Thus, it has great potential in the identification and investigation of pure microglial populations.

To establish the homozygous H9-*TMEM119*-EGFP, we inserted the fluorescent reporter EGFP immediately prior to the *TMEM119* stop codon using the CRISPR/Cas9 system (Fig. 1A). Clones were screened for EGFP insertion through PCR using a primer pair targeted to the edited locus (Fig. 1B). The parental cell line H9 WT was used as a control (Fig. 1B). The forward primer targets outside the donor sequence and the reverse primer targets inside the donor sequence due to the size of the insertion (primers are annotated in Fig. 1A). Clones found to be positive for EGFP insertion, as determined by PCR, were confirmed by sequencing (Fig. 1C). Normal hESC morphology and karyotype were observed after gene targeting (Fig. 1D). The absence of off-target insertions of the donor plasmid was confirmed using sequencing (Fig. S1). The top three coding and top five non-coding sites for off-target insertions were determined using the Benchling online software. H9-*TMEM119*-EGFP hESCs expressed both OCT4 and NANOG in the undifferentiated state, as detected using immunocytochemistry (Fig. 1E). Spontaneous differentiation of hESCs confirmed the ability of the cell line to express markers from all three germ layers using immunocytochemistry (Fig. 1F). Several differentiation kits (STEMCELL Technologies) were used to differentiate hESCs into microglia, based on the protocol described by McQuade and colleagues (Fig. 1G) (McQuade et al., 2018). The cells exhibited the expected morphology throughout the differentiation process (Fig. 1G). EGFP expression was observed in mature microglia (Fig. 1H). Overall, these experiments demonstrated the integrity of the cell line after gene targeting and validated its experimental potential.

3. Materials and methods

3.1. Cell culture

H9-*TMEM119*-EGFP cells were maintained in mTeSR media (STEMCELL Technologies) on growth factor reduced Matrigel (Corning) coated wells and passaged every 5 days or at 70–80 % confluency using

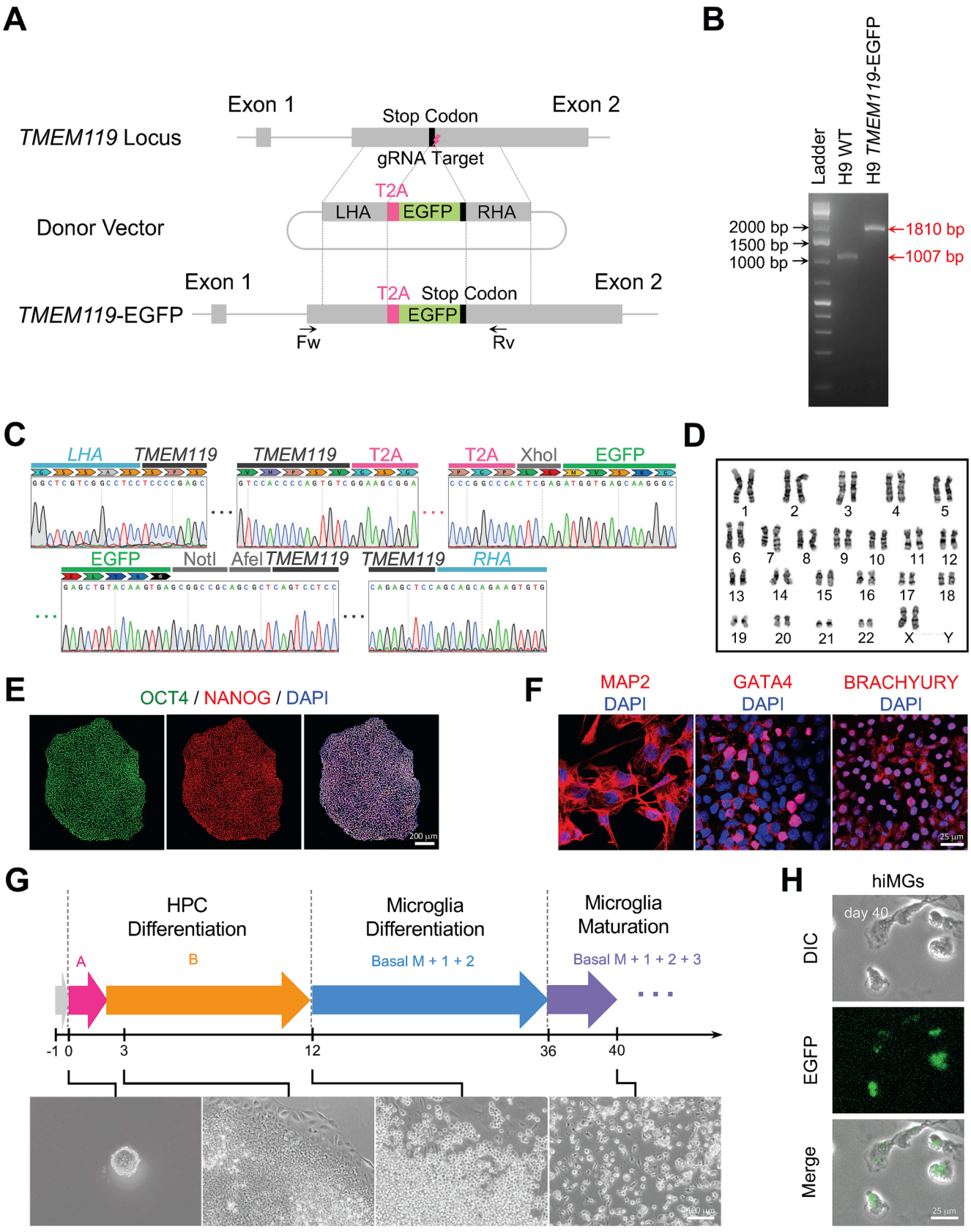
A
TMEM119 Locus
Exon 1
Stop Codon
Exon 2
gRNA Target
T2A
Donor Vector
LHA
EGFP
RHA
Exon 1
T2A Stop Codon
Exon 2
TMEM119-EGFP
Fw
Rv
B
Ladder
H9 WT
H9 TMEM119-EGFP
2000 bp
1500 bp
1000 bp
1810 bp
1007 bp
C
LHA TMEM119
TMEM119 T2A
T2A XhoI EGFP
EGFP NotI AfeI TMEM119
TMEM119 RHA
D
1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 X Y
E
OCT4 / NANOG / DAPI
200 µm
F
MAP2 DAPI
GATA4 DAPI
BRACHYURY DAPI
25 µm
G
HPC Differentiation
Microglia Differentiation
Microglia Maturation
A
B
Basal M + 1 + 2
Basal M + 1 + 2 + 3
-1 0 3 12 36 40
100 µm
H
hiMGs
DIC
EGFP
Merge
day 40
25 µm

Fig. 1.

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Goat anti-OCT4	1:100	Santa Cruz Biotechnology Cat# sc-8628 RRID:AB_653551
	Rabbit anti-NANOG	1:100	Abcam Cat# ab80892 RRID:AB_2150114
3 Germ Layer Differentiation Markers	**Ectoderm**	1:2000	Abcam
	Chicken anti-MAP2		Cat# ab5392 RRID:AB_2138153
	Endoderm	1:300	Santa Cruz Biotechnology
	Mouse anti-GATA4		Cat# sc-25310 RRID:AB_AB_627667
	Mesoderm	1:200	Abcam
	Rabbit anti-BRACHYURY		Cat# ab209665 RRID: AB_2750925
Secondary antibodies	AlexaFluor® 488 donkey anti-goat	1:1000	Molecular Probes Cat# A-11055 RRID:AB_2534102
	AlexaFluor® 488 donkey anti-rabbit	1:1000	Abcam Cat# ab150073 RRID: AB_2636877
	AlexaFluor® 488 donkey anti-mouse	1:1000	Abcam Cat# ab150105 RRID: AB_2732856
	AlexaFluor® 568 donkey anti-rabbit	1:1000	Abcam Cat# ab175470 RRID:AB_2783823
	AlexaFluor® 647 goat anti-chicken	1:1000	Abcam Cat# ab150171 RRID:AB_2921318
Nuclear stain	DAPI	5 ug/ml	Thermo Fisher Scientific Cat# D1306 RRID:AB_2629482
Site-specific nuclease			
Nuclease information	CRISPR/cas9		
Delivery method	Nucleofection		
Selection/enrichment strategy	Puromcyin		
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
Top predicted off-target sites	*DUSP*	ATTATGCCCCCAACACTCAG/ ACTTTCCCGAGCCAAGAAAT	
	AC091814.3	TCCTCTATTCATTGGCTGCTG/ TGGGGAGTCATTGTTTGACA	
	ATP10A	GAGCACTCATCAGGGAGGAC/ AGGCCATTTCAAGGTTTTCA	
	Non-coding	ACTGGGCCCCAAGTAAAAAT/ CAACTGGAACCGTGTGTGTC	
	Non-coding	CTCCCAAAGTGCTGGGATTA/ CTTGGAAAGAGGCTGAGTGG	
	Non-coding	ATCATGCTGTCAGCTCGTGT/ TCTGTGTGCGTACGTGTGTG	
	Non-coding	CAGAGAAGGTGCTGGGTAGC/ CGGACATTGAACAGCTGAGA	
	Non-coding	AGGCACGTCAGTCCACTTCT/ GCTATTGCAGGGCAGTTTCT	
Potential random integration	Plasmid backbone	CTGGCGTAATAGCGAAGAGG/ CGGCATCAGAGCAGATTGTA	
gRNA	*TMEM119* stop codon	CCAGTGTCTAACAGTCCTCC	
LHA Template Amplification (PCR)	pUC19 5′ and T2A 3′ overlap	TCTAGAGGATCCCCGGTACCCTCCCCGAGCCTCCCGCCAC/ CCCCTGCCCTCTCCGCTTCCGACACTGGGGTGGACACTGC	
T2A Amplification (PCR)	*TMEM119* LHA and EGFP 5′ overlap	GCAGTGTCCACCCCAGTGTCGGAAGCGGAGAGGGCAGGGG/ TCCTCGCCCTTGCTCACCATCTCGAGTGGGCCGGGATTTTCCTCCA	
EGFP Amplification (PCR)	T2A 5′ and *TMEM119* RHA overlap	TGGAGGAAAATCCCGGCCCACTCGAGATGGTGAGCAAGGGCGAGGA/ CTGGCAGCCCGGGAGGACTGAGCGCTGCGGCCGCTCACTTGTACAGCTCGTCCATGC	
RHA Template Amplification (PCR)	EGFP and pUC19 3′ overlap	GCATGGACGAGCTGTACAAGTGAGCGGCCGCAGCGCTCAGTCCTCCCGGGCTGCCAG/ CGGCCAGTGAATTCGAGCTCGGTACCAGCGCTGGAGCTCTGAGCACAGGCAG	
Genotyping Primers	Genome–LHA junction	GGTCTGTGCCTGCTACCG	
	LHA	GCCACCCAGAACCTCAAGT	
	EGFP–T2A–LHA junction	GCTGAACTTGTGGCCGTTTA	
	EGFP	CCTGAAGTTCATCTGCACCA	
	EGFP–RHA junction	ACATGGTCCTGCTGGAGTTC	
	RHA–Genome	GCCAAGATTGCACCAAGATT	

ReLeSR (STEMCELL Technologies).

3.2. Gene targeting

CRISPR/Cas9 was used to introduce EGFP into the *TMEM119* locus immediately prior to the stop codon. The gRNA was designed using the Benchling online software (https://www.benchling.com). EGFP was cloned into the pUC19 donor plasmid with left and right *TMEM119* homology arms of 700 bp. 5 μg of the CRISPR/Cas9 gRNA construct, 5 μg of the donor plasmid, and 0.5 μg of the EGFP control plasmid were used to nucleofect 1×10^6 cells with the Nucleofector kit (Lonza). Following nucleofection, cells were maintained in mTeSR media with 10 μM of rock inhibitor. After 24 h, cells were selected using 500 ng/μL puromycin for 2 days. Selected cells were dissociated using TrypLE Express enzyme (Gibco), replated as single cells, and expanded for an additional 10 days in mTeSR media. Clones were manually picked, checked for EGFP insertion using PCR and further validated using sequencing.

3.3. Microglia differentiation

Microglia were differentiated using kits from STEMCELL Technologies (McQuade et al., 2018). Briefly, hESCs were dissociated using ReLeSR and 100 colonies (~50 μm in diameter) were plated on a six-well Matrigel coated (1:5 dilution) plate. Colonies were first differentiated into haematopoietic stem cells (HPCs) before being differentiated to microglia. After 40 days, microglial differentiation was completed, and cells were used for downstream applications.

3.4. Immunocytochemistry

Cells were fixed in 4 % paraformaldehyde for 15 min at room temperature and blocked with PBS containing 3 % bovine serum albumin and 0.1 % TritonX-100 for 1 h at room temperature. They were incubated with primary antibodies (Table 2) overnight at 4 °C. After washing, cells were incubated with secondary antibodies (Table 2) for 1 h at room temperature. All images were captured using a Zeiss LSM880 Airyscan microscope.

3.5. Spontaneous differentiation

hESCs were passaged using ReLeSR and plated on a six-well plate with 2 mL of mTeSR medium per well. After 24 h, medium was changed to differentiation medium (DMEM/F12, 20 % KSR without FGF, 1:100 GlutaMAX, 1:100 NEAA, and 100 μM 2-mercaptoethanol) and replaced every day. After 6–12 days, differentiated cells were stained with germ layer-specific markers. The antibodies used are listed in Table 2.

3.6. Off-Target detection

Potential off-target insertion regions were identified via Benchling (Fig. S1). Primers designed against the top three coding and the top five non-coding regions were used to amplify H9-*TMEM119*-EGFP genomic DNA. The absence of off-target insertions of the donor plasmid was validated by sequencing. The primers used are listed in Table 2.

3.7. Karyotyping

Cells were treated with 10 μg colcemid (Irvine Scientific) per ml of mTeSR media. After 1 h, cells were treated with hypotonic solution for 25 min. Chromosomes were identified according to the International System for Human Cytogenetic Nomenclature and karyogram was made by Cytovision (Leica).

3.8. STR analysis

STR analysis was outsourced and performed by Takara Bio (Japan).

3.9. Mycoplasma

The EZ-PCR Mycoplasma Detection Kit (Biological Industries) was used to test for mycoplasma.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103264.

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